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Maturational arrest of thymocyte development is caused by a deletion in the receptor-like protein tyrosine phosphatase κ gene in LEC rats☆

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Abstract

The Long-Evans Cinnamon (LEC) rat has a spontaneous mutation, *T helper immunodeficiency* (*thid*), which causes a markedly reduced $CD4^+$ thymocyte population. Here we positionally clone the locus and identify a deletion in the gene encoding a receptor-like protein tyrosine phosphatase κ (*Ptprk*) that led to complete loss of the transcript. The rat *Ptprk* gene exhibits 98% identity with the human and mouse counterparts and is expressed most abundantly in the $CD4^-CD8^-$ double-negative stage. The downregulation of *Ptprk* in mouse immature thymocytes by RNA interference mimicked the *thid* phenotype. These results indicate that *thid* maps to the *Ptprk* locus and that functional *Ptprk* is crucial for lineage commitment or progression of $CD4^+$ T cells. We also found that *Ptprk* appears to function in parallel with or downstream of Th-POK/cKrox (also known as ZBTB7B), a master regulator of T cell lineage decision.

Keywords: T cell differentiation; Protein phosphatase; LEC rat; Thymus; Th-POK/cKrox

Mature immunocompetent $CD4^+$ or $CD8^+$ T cells differentiate through a $CD4^+CD8^+$ double-positive (DP) cell stage in the thymus [1]. DP cells express a large number of T cell receptor (TCR) variants through somatic recombination, and signal transduction initiated between the TCR and the major histocompatibility complex (MHC) ligands not only screens out the useful TCRs by negative and positive selection, but also results in a highly specific lineage choice between $CD4^+$ and $CD8^+$ T cells by suppressing either the CD4 or the CD8 molecule [1,2]. This process is remarkable because it is not like other developmentally significant molecular interactions whereby one ligand species typically recognizes one receptor type for proper differentiation. Molecular mechanisms of how T cells choose the correct developmental pathway remain largely unknown.

There are two mutant strains that show T cell developmental defects whereby only $CD4^+$ T cells, not $CD8^+$ T cells, fail to develop properly. The HD mouse is one such mutant [3]. Recently, it was reported that the zinc finger transcription factor, Th-POK/cKrox, is mutated in HD mice [4]. This study, along with another independent finding, suggested that Th-POK/cKrox is a master regulator of lineage commitment [4,5]. However, it is unknown how Th-POK/cKrox expression is regulated or whether the Th-POK/cKrox protein is sufficient for regulating the genes required for the functional maturation of $CD4^+$ T cells.

The Long-Evans Cinnamon (LEC) rat is the other strain that exhibits a reduced $CD4^+$ T cell lineage population [6]. However, the phenotype is not identical to that of HD mice. For example, in the periphery, HD mice lack $CD4^+$ T cells completely, whereas LEC rats have a population of functionally

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defective CD4$^+$ T cells that accumulate in an age-dependent manner [7], which provides a unique opportunity for studying Th-POK/cKrox's role in postlineage decision processes. In the present study we set out to clone the gene for the *T helper immunodeficiency* (*thid*) phenotype positionally in LEC rats and identified *Ptprk,* a member of a family of receptor-like protein tyrosine phosphatases. We found that *Ptprk* appears to function in parallel with or downstream of *Th-POK/cKrox*. Furthermore, our results also indicate that *Th-POK/cKrox* is not sufficient for complete functional maturation of CD4$^+$ T cells.

Results and discussion

Fine-mapping of thid locus and identification of Ptprk gene

The LEC rat has a defect in T cell development, termed *thid,* in which the number of CD4-positive thymocytes is reduced to 5% of that of the control WKAH strain, while the expression of TCR, CD3, CD4, and MHC class II is normal [6]. We have previously determined that *thid* is caused by a single recessive mutation and fine-mapped it to a ca. 30-cM segment on chromosome 1 [8]. The subsequently established congenic strain, WKAH.C-*thid*/Tj, which possesses the corresponding LEC-derived chromosomal region in the WKAH genetic background, showed the *thid* phenotype [9]. The genotyping with newly screened SSLP markers further narrowed the introgressed region to ca. 15 cM between *D1Rat328* and *D1Rat6* on 1p12 (Fig. 1a). To fine-map the region further, WKAH.C-*thid*/Tj rats were crossed to WKAH/Slc to produce *thid*$^{+/-}$ animals, which were then backcrossed to WKAH.C-*thid*/Tj rats to generate a panel of 1007 backcross (N2) rats. All N2 animals were tested for the thymic CD4$^+$ T cell population by FACS analysis and their haplotypes were simultaneously determined (Fig. 1b). The most informative recombinant rat narrowed the location of the *thid* mutation to an interval of ca. 0.5 cM between markers *D1Rat148* and *AU028456* (Fig. 1b).

On the basis of genetic mapping data for the rat and genomic sequence data for the syntenic mouse region available at the time, only two genes, *AHI1–RAT* and *Ptprk* (NP_001025073.1), could be considered candidate genes. PCR amplification of the region in LEC and WKAH.C-*thid*/Tj strains failed for the *Ptprk* gene, suggesting a major genomic deletion, which was subsequently confirmed by Southern blot analysis (Fig. 1c). We found that in the LEC genome, at least the region corresponding to exon 4 to exon 12, was deleted, indicating that the breaking point of *Ptprk* exists between exon 3 and exon 4 (Fig. 1d). No other genomic deletion was found in any other segment within the mapped region, including the *AHI-1–RAT* gene (data not shown). Consistent with the mapping data that the

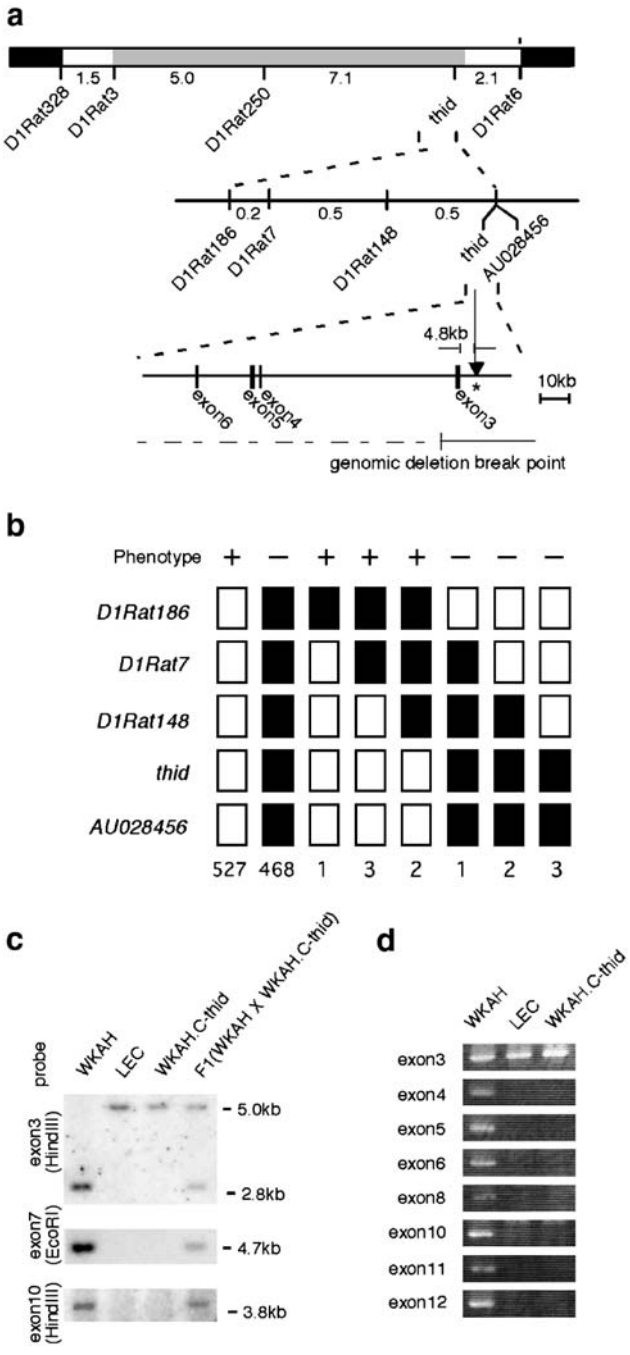

Fig. 1. Genetic linkage mapping of *thid* on rat chromosome 1. (a) Genetic linkage map of chromosome 1. The top bar is a schematic of the 1p12 region showing the WKAH.C-*thid* congenic strain. The known extent of the LEC-derived portion is designated by the gray bar. The open ends designate intervals containing the recombinant points. The black boxes represent the region of the WKAH rat, host strain. Shown below the bar are recombination frequencies (cM). The arrow indicates the location of the *AU028456* marker. (b) Haplotype analysis of *thid* recombinants. Distribution of haplotypes from chromosome 1 in 1007 animals from the (WKAH.C-*thid* X WKAH) × WKAH.C-*thid* backcross is shown. The phenotype of these progeny was determined as described under Materials and methods. The "plus" indicates wild-type thymus development, the "minus," the *thid* phenotype, i.e., reduced CD4 thymus population. Black boxes indicate homozygosity for the LEC allele, white, heterozygosity. The *thid* locus is described as the LEC allele when the deletion is found in the *Ptprk* gene. Each column represents a chromosome and the number below indicates the frequency in the crosses. We did not detect any recombinant between the *Ptprk* gene mutation and the *thid* phenotype. (c) Southern blot analysis of the *Ptprk* region revealed that the breaking point resides within a few kilobases in proximity to exon 3. Genomic deletion was confirmed for regions corresponding to exon 7 and exon 10 of the *Ptprk* gene. (d) PCR amplification failed for genomic regions corresponding to exons 4, 5, 6, 8, 10, 11, and 12 of *Ptprk.* Combined with Southern hybridization data, the deletion removes from exon 4 to at least exon 12, corresponding to amino acid 159 to 718.

AU028456 marker and *thid* were tightly linked, the physical distance between the 5′ end of the third exon and *AU028456* was only 4.8 kb, revealing that *AU028456* was part of the intron of the *Ptprk* gene itself. Thus, we considered *Ptprk* the most significant candidate. Next we determined the full-length cDNA using the RACE method. The rat *Ptprk* sequence was found to be highly homologous to human and mouse with 98% identity (data not shown) [10,11].

Northern blot analysis confirmed that the mRNA of *Ptprk* was not detectable in WKAH.C-*thid*/Tj and LEC rat thymus as inferred from the major genomic deletion (Fig. 2a). *Ptprk* mRNA is expressed in thymus as well as in many other tissues, which is consistent with a mouse EST database (Fig. 2b).

Functional assay of Ptprk with FTOC (fetal thymus organ culture) analysis

To confirm that loss of function of PTPRK is responsible for the *thid* phenotype, we performed an RNAi-mediated gene knockdown assay using mouse FTOC. First we examined the specificity of the short hairpin RNA (shRNA) by showing that only when the target shRNA-encoded virus was transduced was the reduction of the reporter protein observed (Fig. 3a). When endogenous *Ptprk* expression is downregulated using the same target shRNA in the fetal thymocytes, we found that these *Ptprk*^{low} T cells failed to develop into the CD4$^+$ T cell lineage (Fig. 3b). Although the reduction in the CD4$^+$ T cell population is milder compared to LEC, the percentage of the CD4$^+$ T cells dropped to one-fourth of that of control (Fig. 3c). This result indicated that the function of PTPRK is required in T cells rather than thymic stromal cells, as we showed previously [12,13].

Ptprk is crucial for functional maturation of CD4$^+$8$^-$ T cells in the thymus

We next utilized quantitative PCR to track the expression profile of *Ptprk* during the course of T cell development in wild-type rats. *Ptprk* was expressed in CD4$^-$CD8$^-$ T cells, but

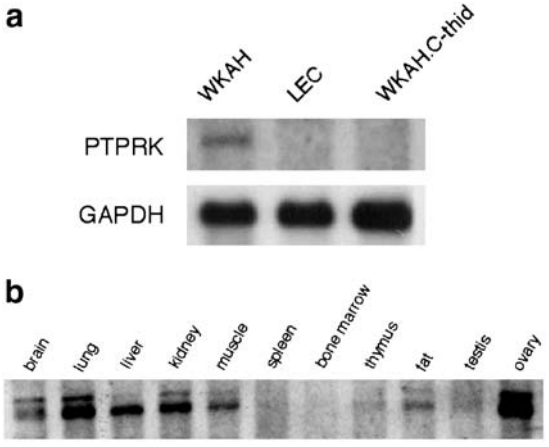

Fig. 2. Northern blot analysis of the *Ptprk* transcript. The blot contained 1 μg of poly(A)$^+$ RNA from (a) thymus or (b) the indicated tissues derived from the control WKAH strain . The filter was hybridized with a probe for exon 3 of *Ptprk*.

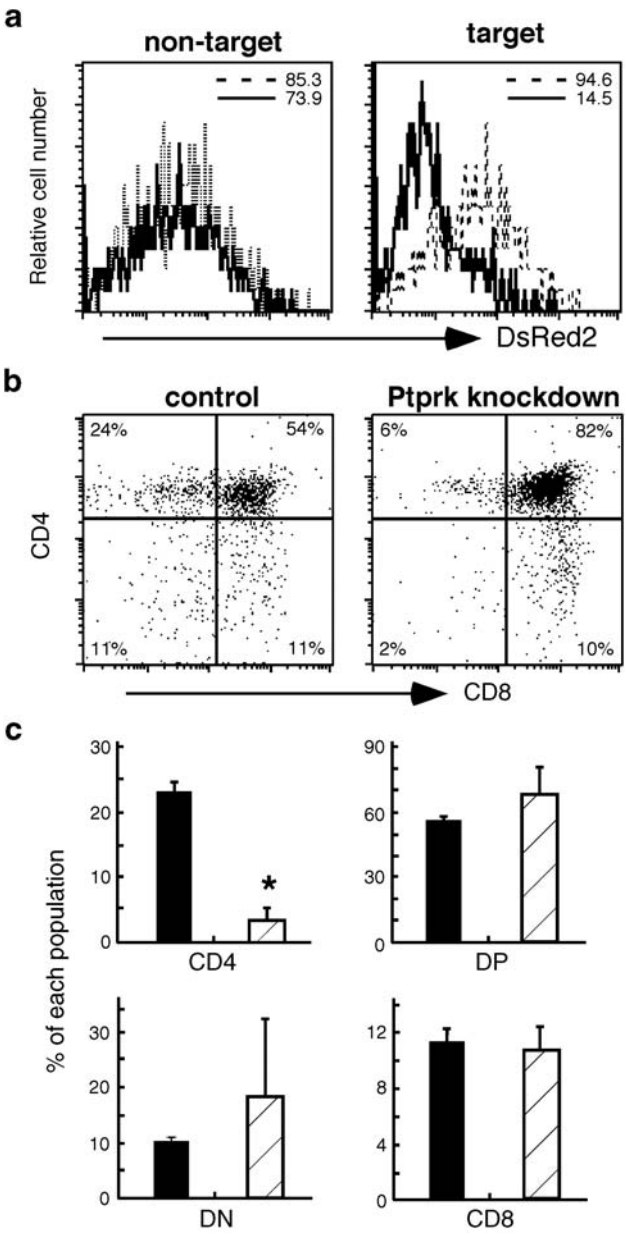

Fig. 3. Downregulation of *Ptprk* inhibits the development of CD4 single-positive cells. (a) Efficiency of shRNA for mouse *Ptprk*. DO11.10 T cell hybridomas were transduced with the virus vectors encoding DsRed2 with RNAi-targeted *Ptprk* sequence (right) or nontargeted sequence (left) in the 3′ UTR. The cells were further transduced with the virus vectors encoding shRNA for *Ptprk* (solid line) or empty vector (dotted line). The numbers in the top right corners are mean fluorescence intensity of DsRed2. (b) Downregulation of *Ptprk* inhibits the development of CD4 single-positive cells in fetal thymus organ culture. Fetal thymocytes were transduced with the virus vector encoding shRNA for *Ptprk* or empty vector, as described under Materials and methods. The numbers in each quadrant are the percentages of each population. (c) Statistical analysis of the effects of *Ptprk* shRNA on thymocyte differentiation. Filled and hatched bars represent control and *Ptprk* knockdown cell populations, respectively. The results are expressed as means with SD ($n=3$). *$p<0.001$.

expression declined in DP cells and reactivated in the CD4$^+$, but not in the CD8$^+$, single-positive subset in the thymus. In the periphery, *Ptprk* expression was diminished again in CD4$^+$ T cells (Fig. 4a). These results suggest that the protein function is required for lineage choice and that the process of reactivation

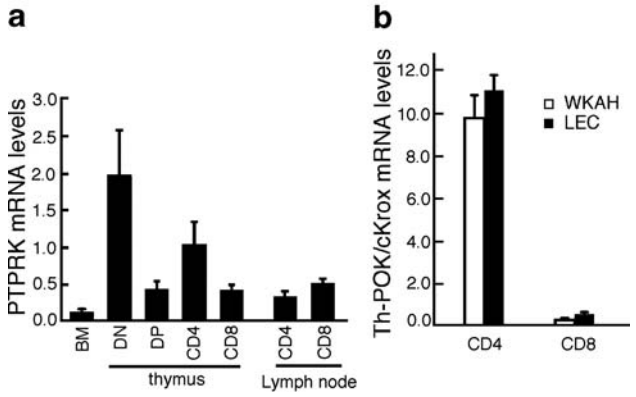

Fig. 4. *Ptprk* expression profile during T cell development. (a) *Ptprk* expression profile during T cell development within thymus and lymph node. BM, bone marrow; DN, double negative; DP, double positive; CD4, $CD4^+$ single positive; CD8, $CD8^+$ single positive cells. (b) Peripheral mRNA levels of *Th-POK/cKrox* were compared between WKAH and LEC rats. Relative expression levels, normalized to *Gapdh* levels, are shown. Data are presented as means ± SD ($n=4$).

in $CD4^+8^-$ thymocytes is a pivotal point for functional maturation of $CD4^+8^-$ single-positive T cells in the thymus.

We also examined the transcript levels of several genes whose functions have been reported to be important for T cell development, namely *CD4, CD8A, CD8B, CD3, Runx3, Gata3, Notch1,* and *Lck.* However, none of these genes in thymocytes changed expression levels in *Ptprk*-deficient strains (data not shown). The Th-POK/cKrox transcription factor is a recently found key regulator of $CD4^+$ T cell development [4,5]. The mRNA of *Th-POK/cKrox* in total thymocytes of the LEC rat was significantly reduced, but this is not surprising because it merely reflects the fact that in LEC rats there is a substantial reduction of $CD4^+$ thymocytes, which are the only subtype expressing *Th-POK/cKrox* (data not shown). Therefore, we tested the $CD4^+$ T cells that accumulate with age in the lymph node of LEC rats [7]. The peripheral $CD4^+$ T cell population in the LEC rat is restored with age to nearly 40 to 60% of that of wild-type rats, but these $CD4^+$ T cells can neither produce IL-2 or IL-4 cytokines in response to mitogens nor respond to T-cell-dependent antigens in vivo [9,14]. Therefore, these residual $CD4^+$ T cells are functionally defective. We found that *Th-POK/cKrox* expression was equal between the control and the LEC rat (Fig. 4b). This finding indicates that Th-POK/cKrox is not sufficient for complete functional maturation of $CD4^+$ T cells and that PTPRK is crucial not only for producing $CD4^+$ T cells but also for endowing them with functional capability. In addition, *Ptprk* does not influence the expression of *Th-POK/cKrox*, at least in the periphery. Thus, *Ptprk* is likely to function in parallel with or downstream of *Th-POK/cKrox*. It is also worth mentioning that *Ptprk* expression is downregulated at the DP stage and upregulated again in the $CD4^+$ SP stage (Fig. 4a). This might reflect the fact that the first induction is necessary for the proper lineage decision, while the second is necessary for subsequent maturation.

Several groups, including ours, reported that lineage choice between $CD4^+$ and $CD8^+$ T cells is determined by the strength or duration of TCR signaling [15–17]. The thymocytes that receive a moderate, prolonged TCR signal choose a $CD4^+$ fate, while those that receive a weak, transient TCR signal become $CD8^+$ single-positive T cells [15]. Thus it is intriguing to speculate that PTPRK enhances TCR signaling by dephosphorylating substrate proteins. It is also possible that *Ptprk* would be involved in the selective survival of $CD4^+$ T cells rather than the $CD4^+$ T-cell-lineage specification process. The use of *Ptprk* gene knockout mice, which are under construction, and analysis of the substrate protein of *Ptprk* would provide further insight into the mechanisms of how *Ptprk* controls $CD4^+$ T cell development.

Materials and methods

Animals

LEC/Tj and congenic strain WKAH.C-*thid*/Tj rats have been previously described [6,9]. Briefly, the congenic strain was established by eight successive backcrosses (N8) followed by intercrosses to establish homozygous strains and had been maintained for 38 generations (N8F38). All animals were bred in the Institute for Animal Experimentation of the University of Tokushima under SPF conditions. WKAH/Slc rats and pregnant C57BL/6 mice were purchased from Japan SLC. Animal procedures used in this study were approved by the University of Tokushima Animal Experimentation Committee.

Molecular analyses—Northern/Southern blot and RACE

Total RNA from thymus was extracted with TRIzol (Life Technologies). One microgram of poly(A) RNA was separated on a 2% agarose gel, and DIG-labeled probes against exon 3 of *Ptprk* were used. RACE was performed using the SMART RACE cDNA amplification kit (Clontech). Primers were designed from partial cDNA sequences on the Rat Ensembl database, which were 5′-GAGCAGGACGCGAATCTCGTATTCTGTA-3′ and 5′-GAGCCCCAAGCTTACCTGACTATGAAGG-3′ for 5′- and 3′-RACE, respectively. The obtained products were cloned into the pGEM-T Easy vector (Promega) and sequenced. Sequence data were deposited with DDBJ (Accession Nos.AB288087 and AB288088).

Construction of shRNA-expressing vector

A fragment of IRES-EGFP in the pKE004 retrovirus vector [18] was replaced by EGFP from pEGFP-N3 (BD Clontech). In the 3′ UTR region of this vector, oligonucleotides encoding shRNA for *Ptprk* were inserted with the miR-30 fragment [19]. The inserted oligonucleotide sequence was 5′-TGCTGTTGACAGTGAGCGCCGTCACTATCTGCTACCATTATAGTGAAGCCACAGATGTATAATGGTAGCAGATAGTGACGTTGCCTACTGCCTCGGAGC-3′. The construction was confirmed by DNA sequencing.

Virus production and transduction

The retroviral vector constructs were transfected into the retrovirus-packaging cell line Plat-E [20]. Briefly, 1×10^6 cells/well were seeded into a six-well plate in 1.5 ml DMEM supplemented with 10% fetal bovine serum, 0.05 mM 2-mercaptoethanol, and 100 mg/ml penicillin/streptomycin. Subconfluent cells were transfected with 1 mg plasmid DNA and 5 ml Fugene 6 (Roche Molecular Biochemicals, Mannheim, Germany). The next day, the medium was refreshed, and on the following day the supernatant was harvested and passed through a 0.45-mm syringe filter.

For retrovirus transduction, the retroviral supernatant was added to cells with 10 mg/ml Polybrene (Chemicon International, Temecula, CA, USA). Retroviral infection was facilitated by centrifugation at 2600 rpm for 1 h.

Check of RNAi efficiency

DO11.10 T cell hybridoma cells were transduced with the virus vector encoding DsRed2 with *Ptprk* RNAi-target (CACACTTTCAACGTCACTAT-

CTGCTACCATTACTTCCGTGGC) or nontarget (GTGGAAGTTGGCCGG-GTGAAATGCTATAAATATTGGCCTGAT) sequences in the 3′ UTR. After 1 week, DsRed2-positive cells were sorted by a JSAN cell sorter (Bay Bioscience) and further transduced with the virus vector encoding the shRNA for *Ptprk.* Two days later, the expression of DsRed2 in GFP^+ cells was assessed by FACSCalibur (BD Biosciences).

Fetal thymus organ culture

Fetal thymic lobes were obtained from fetal day 15 C57BL/6 embryos. Cell suspensions were prepared from the thymic lobes and transduced with the retrovirus vector as described above. After cells were cultured for 1 day in medium supplemented with 5 ng/ml Flt3L and 5 ng/ml IL-7, GFP^+ cells were sorted by a JSAN cell sorter (Bay Bioscience). The sorted cells were aliquoted at 2000 cells/well in Terasaki plates and one dGuo-treated (7 days) thymic lobe per well was added. The cells and lobes were incubated for 24 h as hanging drop cultures and then placed on the Nucleopore Track-Etch membrane (Whatman, Clifton, NJ, USA) in 24-well plates for 10 days [21]. The differentiation of these thymocytes was analyzed by FACSCalibur (BD Biosciences).

Quantitative polymerase chain reaction

After DNase treatment, total RNA was reverse-transcribed using $oligo(dT)_{20}$ primers and ThermoScript RT (Invitrogen) to obtain cDNA. Quantitative PCR was done on the Opticon 2 (Bio-Rad) using the DyNAmo HS SYBR Green qPCR with ROX kit (Finnzymes). All assays were done at least four times and normalized to *Gapdh.* Primer sequences are available upon request.

Acknowledgments

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References

[1] R.N. Germain, T-cell development and the CD4–CD8 lineage decision, Nat. Rev., Immunol. 2 (2002) 309–322.
[2] C.A. Janeway Jr., The T cell receptor as a multicomponent signalling machine: CD4/CD8 coreceptors and CD45 in T cell activation, Annu. Rev. Immunol. 10 (1992) 645–674.
[3] V.P. Dave, D. Allman, R. Keefe, R.R. Hardy, D.J. Kappes, HD mice: a novel mouse mutant with a specific defect in the generation of CD4(+) T cells, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 8187–8192.
[4] X. He, et al., The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment, Nature 433 (2005) 826–833.
[5] G. Sun, et al., The zinc finger protein cKrox directs CD4 lineage differentiation during intrathymic T cell positive selection, Nat. Immunol. 6 (2005) 373–381.
[6] T. Agui, et al., Maturational arrest from $CD4^+8^+$ to $CD4^+8^-$ thymocytes in a mutant strain (LEC) of rat, J. Exp. Med. 172 (1990) 1615–1624.
[7] T. Yamada, et al., Inheritance of T helper immunodeficiency (thid) in LEC mutant rats, Immunogenetics 33 (1991) 216–219.
[8] K. Wei, Y. Muramatsu, T. Sakai, T. Yamada, K. Matsumoto, Chromosomal mapping of the T-helper immunodeficiency (thid) locus in LEC rats, Immunogenetics 47 (1997) 99–102.
[9] T. Sakai, et al., Unresponsiveness of $CD4^-8^{+/-}$thymocytes to lectin stimulation in LEC mutant rats, Immunology 95 (1998) 219–225.
[10] Y.P. Jiang, et al., Cloning and characterization of R-PTP-kappa, a new member of the receptor protein tyrosine phosphatase family with a proteolytically cleaved cellular adhesion molecule-like extracellular region, Mol. Cell. Biol. 13 (1993) 2942–2951.
[11] Y. Yang, et al., Molecular cloning and chromosomal localization of a human gene homologous to the murine R-PTP-kappa, a receptor-type protein tyrosine phosphatase, Gene 186 (1997) 77–82.
[12] T. Agui, T. Sakai, K. Himeno, K. Matsumoto, Bone marrow-derived progenitor T cells convey the origin of maturational arrest from $CD4^+CD8^+$ to $CD4^-CD8^+$ thymocytes in LEC mutant rats, Eur. J. Immunol. 21 (1991) 2277–2280.
[13] T. Sakai, T. Agui, K. Matsumoto, Maturational arrest from $CD4^+8^+$ to $CD4^+8^-$ thymocytes is caused by the bone marrow-derived cells in LEC mutant rats, Immunol. Lett. 38 (1993) 145–152.
[14] T. Sakai, T. Agui, K. Matsumoto, Abnormal CD45RC expression and elevated CD45 protein tyrosine phosphatase activity in LEC rat peripheral CD4+ T cells, Eur. J. Immunol. 25 (1995) 1399–1404.
[15] K. Yasutomo, C. Doyle, L. Miele, C. Fuchs, R.N. Germain, The duration of antigen receptor signalling determines $CD4^+$ versus $CD8^+$ T-cell lineage fate, Nature 404 (2000) 506–510.
[16] E.O. Matechak, N. Killeen, S.M. Hedrick, B.J. Fowlkes, MHC class II-specific T cells can develop in the CD8 lineage when CD4 is absent, Immunity 4 (1996) 337–347.
[17] L.L. Sharp, D.A. Schwarz, C.M. Bott, C.J. Marshall, S.M. Hedrick, The influence of the MAPK pathway on T cell lineage commitment, Immunity 7 (1997) 609–618.
[18] Y. Maekawa, et al., Delta1–Notch3 interactions bias the functional differentiation of activated $CD4^+$ T cells, Immunity 19 (2003) 549–559.
[19] J.M. Silva, et al., Second-generation shRNA libraries covering the mouse and human genomes, Nat. Genet. 37 (2005) 1281–1288.
[20] S. Morita, T. Kojima, T. Kitamura, Plat-E: an efficient and stable system for transient packaging of retroviruses, Gene Ther. 7 (2000) 1063–1066.
[21] T. Ueno, C. Liu, T. Nitta, Y. Takahama, Development of T-lymphocytes in mouse fetal thymus organ culture, Methods Mol. Biol. 290 (2005) 117–133.